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Growth benefits provided by different arbuscular mycorrhizal fungi to *Plantago lanceolata* depend on the form of available phosphorus

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ABSTRACT

Strains of arbuscular mycorrhizal fungi (AMF) differ markedly in the growth benefits they provide to plants. We investigated whether these differences depend on the chemical form of inorganic phosphorus. The closely related AMF *Glomus custos* and *Rhizophagus irregularis* were compared using *Plantago lanceolata* as the host plant, grown in quartz sand with either soluble orthophosphate or sparingly soluble hydroxyapatite as a sole source of phosphorus. In a growth experiment with AMF-inoculated plants in a climate chamber, sampling at 3-wk intervals enabled a detailed time-resolved analysis of shoot and root phosphorus concentrations and growth performance of *P. lanceolata*. The ability of AMF to enhance plant growth and deliver phosphate depended strongly on the identity of the available phosphorus source. In orthophosphate-amended substrate only modest differences in plant growth performance (dry matter accumulation and allocation, phosphorus acquisition) were observed between the two AMFs, despite evident AMF root colonization as shown by strain-specific mtLSU qPCR analysis. The treatment with hydroxyapatite however, created stringent growth-limiting conditions and significantly increased the growth benefit provided by *R. irregularis* over *G. custos* and the non-mycorrhizal treatment. Plants with *R. irregularis* could acquire much more phosphorus from apatite compared to *G. custos*. There were also differences in shoot-to-root dry matter allocation and plant tissue phosphorus concentrations between the *R. irregularis* and *G. custos* treatments. Our observations suggest that in experiments on the symbiosis between plants and mycorrhizal fungi more attention should be paid to the chemical form of phosphorus in soil.

1. Introduction

Arbuscular mycorrhizal fungi (AMF) are important root symbionts in the majority of terrestrial plants because of their ability to enhance nutrient uptake [1]. It is generally accepted that both mycorrhizal and non-mycorrhizal plants rely on orthophosphate in the soil pore water that is exchanged with phosphorus complexes in the soil [2,3]. Due to the very low mobility of phosphate (P) in solution, replacement does not usually keep pace with local uptake, leading to so-called depletion zones in the rhizosphere [4]. The effectiveness of AMF to increase phosphate acquisition of their hosts can largely be attributed to the ability of their hyphae to grow beyond these depletion zones with greater ease than plant roots, thus scavenging larger volumes of soil for P [5].

It is well established that AMF strains show a large functional diversity [6–9], with some fungi appearing to transfer less P to the host than others. Indeed, AMF have been shown to differ in the efficiency with which they explore soil, and this has been related to differences in morphology of the extraradical mycelium (ERM), the density and extension of the ERM network in the soil, the efficacy of the mycorrhizal pathway in the uptake of P (density and K_m value of P_i-transporters) and the transfer of P to the host root [7,10–12]. Apart from the differences in soil exploration efficiency, other factors, biotic as well as abiotic, may determine the benefits for the plant. For example, the carbon to phosphate exchange ratio between the host plant and its fungal partner is important in explaining the variation in host carbon investments for specific plant-fungus combinations [8,13–15]. Likewise, the interaction between light and soil nutrients can affect preferential bidirectional allocation patterns of C and P [16,17]. As a result, reported effects of AMF on plant growth fall within a broad range of outcomes with fungal symbiont qualifications varying from 'high quality' to 'antagonistic' [18].

However, little is known about how mycorrhizal 'quality' is determined by the ability of the fungus to access and utilize different forms of P present in soils. Along with organic P pools [19,20], sparingly soluble (crystalline) P minerals such as apatites ($Ca_{10}X(PO_4)_6$, where $X = F^-$, Cl^-, OH^- or CO_3^{2-}), may contribute significantly to the total phosphorus content of a soil, up to 75–90%, especially in the

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more calcareous soil types [21–24]. These Ca-phosphate species are often denoted by the term rock phosphates if they originate from a marine diagenetic mineral deposit [4,22]. A number of plant-mycorrhiza studies have demonstrated that AMF can successfully mobilize the phosphorus in apatites or at least, can significantly contribute to the solubilization of these P minerals, *e.g.*, *Glomus manihotis* [25]; *Glomus fasciculatus* and *Glomus tenuis* [26], *Glomus clarum* [27] and *Glomus margarita* [28]. In root organ cultures it was shown that the extraradical mycelium (ERM) of *Rhizophagus irregularis* (formely known as *Glomus intraradices*) was able to release orthophosphate from hydroxyapatite, and to a lesser extent, also from a low-reactive rock phosphate mineral [29]. So while P acquisition is clearly important for mycorrhizal species, there have been no quantitative comparisons of how the ability to mobilize phosphate from apatite affects the quality of AMF to their hosts.

Our aim was therefore to establish if the ability to release P from a resilient apatite matrix differed between the two closely related fungal species *Glomus custos* and *Rhizophagus irregularis*. We were interested in a comparison between these species because in previous research we found that *G. custos* was a poorer quality symbiont compared to *R. irregularis* (~24% reduction in shoot dry weight and shoot P content of *Plantago lanceolata* [30]. This lower quality has been confirmed with respect to vegetative growth and P status of a diversity of hosts, including *Allium porrum* and *Medicago truncatula* [13], and *Glycine max* [31]. However, these studies used readily available phosphate species such as orthophosphate as a P source. In this study we wanted to investigate whether the differential beneficial properties of AMF were also evident when using a more natural, recalcitrant P source, such as apatite. Since the reactivity of phosphate in apatites largely depends on the (variable) level of residual carbonate in the crystal lattice [32,33] we used in this study a well-defined model apatite: a synthetically produced crystalline/crystallite hydroxyapatite mineral ($Ca_{10}(PO_4)_6(OH)_2$ rather than rock phosphate [34,35].

An important aspect of characterising the functional diversity in fungal species is the time of harvest in relation to the growth cycle of the plant. In most studies of arbuscular mycorrhizal systems, conclusions are based upon a single harvest because of experimental constraints. This means that critical changes in the life-cyle of the plant may go unnoticed [36]. In this study we monitored the P acquisition ability of the two AMF species in a time course experiment by harvesting *P. lanceolata* at 3-week intervals over a period of approximately 4 months. We hypothesized that the quality differences between *G. custos* and *R. irregularis* noticed in previous studies can be attributed to – at least partly – the differential capabilities of these strains to solubilize the more recalcitrant inorganic P species present in (calcareous) natural soils. In addition to total P acquisition by *P. lanceolata*, other parameters reflecting the impact of the AMF on the host plant were also monitored, *i.e.*, dry matter yields with the shoot-to-root mass allocation ratio, and the tissue P concentrations in shoots and roots. Quantitative PCR (qPCR) using strain-specific mtLSU DNA markers was applied to assess and compare the relative abundances of *R. irregularis* and *G. custos* biomass in the roots within and between the two time course experiments.

2. Materials and methods

2.1. General experimental setup

Two experiments were conducted, one using orthophosphate and the other using apatite as sources of phosphorus. Each involved three different AMF inoculation treatments and 120 pots, of which 7 (1st experiment) or 8 (2nd experiment) replicates per treatment were sacrificed every 3 weeks over a period of 15 weeks. Because the available climate chamber facility did not provide sufficient bench space to accommodate them simultaneously the experiments were conducted consecutivily. This was considered a minor constraint since growth conditions were stringently controlled and closely reproduced with respect to temperature, air humidity, mineral nutrients supplement regime and seed germination (see 2.3).

2.2. Growth substrate and nutrient additions

Pure high-grade quartz sand, autoclaved at 120 °C, ($SiO_2 \geq 99.7\%$, P-free, S60 size fraction 150–300 µm) from Sigrano BV Maastricht, Netherlands, was applied as the sole growth substrate. No constituents other than quartz sand were used in order to maintain defined growth nutrient conditions with respect to the available P source. Also, the substrate was not microbially inoculated with a suspension of native soil (the so-called 'microbial wash'), because such extracts may contain colloidal or fine-sized Ca-phosphate conglomerates [37,38].

Plants were grown in 0.88 l plastic pots (11 cm diameter) with 750 g substrate, which was maintained at a 15% (w/w) water content by weighing. In the orthophosphate treatment, a ½-P strength pH 6.0 Hoagland nutrient solution [39] was added once every two weeks at a rate of 4 ml/kg wet quartz sand, starting at t = 0 (wk0) right after the transplanting of seedlings. The nutrient solutions were injected below the quartz sand surface (over a depth of approximately 1–6 cm) at four equidistant positions around the root (~2 cm away from the pot wall) to achieve an even distribution of the nutrients over the pot volume. This resulted in a cumulative amount of 24 µmol P per pot over the course of the experiment. This rate of mineral nutrient supplementation was similar to that in previous mycorrhiza studies [30].

In the hydroxyapatite experiment, fine-powdered $Ca_{10}(PO_4)_6(OH)_2$ (purity ≥ 99.9%; Acron Organics, Belgium) was thoroughly mixed with quartz sand (1 g per kg sand, equivalent to ~3.8 mmol P per pot) prior to moistening and autoclaving. The level of hydroxyapatite was chosen based on the concentration range of low soluble P minerals encountered in calcareous dune soils of the so-called Renodunaal coastal district in the Netherlands [40], and reported apatite contents measured in other (non-cultivated) lime-rich soils [23,24]. Pots in the hydroxyapatite treatment received mineral nutrients at the same rate as in the orthophosphate treatment, but with all phosphate omitted from the Hoagland nutrient solution. In these modified nutrient solutions, $NH_4H_2PO_4$ was substituted by an equimolar amount of NH_4NO_3.

The starting pH of the substrates was in the range of 7.8–7.9, irrespective of amendment with soluble orthophosphate or the solid hydroxyapatite. Bulk substrate pH values were measured at each timepoint before harvesting shoot and root of the pots.

2.3. Plant culturing and mycorrhizas

Plantago lanceolata L. (Ribwort Plantain) seeds were obtained from a large *c.* 5-year old stock collection, originally collected from open field (low fertile) grown plants (Cruydthoek, Assen, the Netherlands), and thereafter permanently stored in the dark at 4–5 °C. Seeds were sterilized using diluted bleach (2.5% NaOCl) for 10 min, washed with sterilized water and germinated on quartz sand (17% w/w water content) under transparent plastic foil at 20 °C in the climate chamber also used for the P addition treatments (see below). Two weeks after sowing, one randomly selected seedling was planted in the middle of each pot. Roots of the seedlings were inoculated with liquid spore material procured from *in vitro* root organ culture by Mycovitro S.L. Biotechnología Ecológica (Granada, Spain). The spore suspension also contained some hyphae and *Daucus carota* root parts. Inoculation treatments were as follows: (1) *Rhizophagus irregularis*, strain 09 [41], previously *Glomus intraradices*, (2) *Glomus custos*, strain 010 [42], and (3) a heat-sterilized (120 °C) 50:50 mixture of *R. irregularis* and *G. custos* as the non-mycorrhizal (control) treatment. In all cases, a total of approximately 1000 spores were applied.

Pots were randomly positioned on the bench and reshuffled two times a week upon watering. Five destructive samplings, at 3 week intervals, were conducted with the first harvest 3 weeks after planting.

As a proxy for the 0-wk time point in the time course growth series we used 120 lyophilized 2-wk old seedlings to determine an average dry weight of shoot and root, and their respective tissue P contents, on a per seedling basis.

The experiments were conducted in a climate-controlled chamber with a day-temperature of 20 °C, a night-temperature of 18 °C and a relative air humidity of ~75%. The climate chamber provided a day-night light cycle of 14–10 h with a photosynthetically active radiation (PAR) of *c.* 175–185 μmol m^{-2} s^{-1} (Philips day light TL-D no. 480 tubes) at the level of the pot surfaces. Although irradiance was quite homogenous throughout the climate chamber, pot positions were frequently randomly rearranged (see above). The light climate in the room was regularly checked (12 fixed positions on each of two benches) using a PAR quantum photometer (model LI-185 B, LI-COR inc., USA). The average light intensity during orthophosphate experiment with was 186 ± 4.3 (range: 170–210) μmol m^{-2} sec^{-1}; in the hydroxyapatite experiment it was 174 ± 4.5 (range: 155–190) μmol m^{-2} sec^{-1}.

The quartz sand surface of each pot was covered with plastic foil to reduce evaporation, and to minimize the chances of microbial contaminations from the air. Introduction of non-sterile biological materials into the climate chamber was prevented to reduce the risks of contamination and care was taken not to inflict (cross)contaminations of the pots by nutrient injections.

2.4. Shoot and root sampling

At each sampling shoots were removed from the pots, their dry weights (DWs) determined after freeze-drying, and stored at −80 °C. Roots were washed on sieve (0.5 mm mesh) to gently remove most of the quartz sand, thus enabling a direct microscopic examination of the roots for the presence of vesicles/arbuscules and attached ERM (with spores) of the AMF, or for their absence in the case of the non-mycorrhizal controls. After this inspection the roots were thoroughly washed on the sieve under running water to remove adhering quartz grains, the DWs determined after freeze-drying, and the lyophilized biomass stored at −80 °C. Dry matter allocation to the shoot and root system was expressed as the root mass fraction, calculated according to: Root mass fraction = Root DW/(Root DW + Shoot DW).

2.5. Chemical and molecular analyses

Freeze-dried roots and shoots were ground to powder using a metal lockable tube and a metal bullet for 30 s at the highest speed (30 strokes/s; Retsch MM200, Retsch GMBH, Haan, Germany), and stored again at −80 °C until chemical and molecular analysis. The P concentration of the ground shoot and root tissue samples was determined with the molybdate blue ascorbic acid method following acid digestion [43]. The P content of shoot and root systems was calculated on a per plant basis (*i.e.*, plant organ tissue P concentration x DW plant organ). P allocation to the shoot and root system was expressed as the root P fraction, calculated according to: Root P fraction = Root P content/(Root P content + Shoot P content).

Fungal DNA was extracted from approximately 5 mg ball-milled dry root material using DNeasy Plant Mini Kit (Qiagen) following the manufacturer's recommendations with DNA eluted in 50 μl elution buffer. The abundance of the two AM fungal species in the different samples was quantified by quantitative PCR, using taxon-specific markers with hydrolysis probes [13,44]. Plasmid DNA of the cassava mosaic virus (CMV) was used as an internal standard ($1*10^9$ copies of plasmid DNA added in step one of the Plant Mini Kit protocol). The PCR targeted the gene encoding the mitochondrial large sub-unit ribosomal RNA (mtLSU) of *R. irregularis* using the following sense primer: 5′-TTTTAGCGATAGCGTAACAGC-3'; antisense primer: 5′-TACATCTAG GACAGGGTTTCG-3'; and TaqMan probe: 5′-AAACTGCCACTCCCTCCA TATCCAA-3'. Reactions were run on a CFX96 Touch™ Real-Time PCR Detection System, using the following thermal cycling program: Denaturation for 3 min at 95 °C followed by 39 cycles of 5 s at 95 °C, annealing for 10 s at 58 °C, and amplification for 1 s at 72 °C, using 4 μl of template, 5.28 μl of water, 0.32 μl of each primer, 0.048 μl of TaqMan probe and 10 μl of BioRad iTaq™ Universal Probe Supermix.

G. custos mtLSU abundance was monitored using the following sense primer: 5′-TCTAACCCCAGAAATGTATAG-3'; antisense primer 5′-AAG GACTGCCTTGTGTTC-3'; and TaqMan probe: 5′-ATACAATAATGGGCA ATCAGACATATCGT-3'. The qPCR thermal cycling followed the program: Initial denaturation for 5 min at 95 °C followed by 39 cycles of 10 s at 95 °C, 15 s at 62 °C and 1 s at 72 °C, ended by cooling for 30 s at 40 °C.

Standard curves were produced using a dilution series of plasmids containing the mt LSU or the CMV fragment. The standard curves were subsequently used to convert Cq values to mtLSU gene copy numbers. The DNA isolation efficiency was calculated by dividing the copy number of the internal standard in the sample by the initial copy number of the internal standard at the moment of spiking. Then, correcting the raw *R. irregularis* and *G. custos* copy numbers, the arbuscular mycorrhizal abundance (expressed as the logarithm of mtLSU rRNA gene copy number per mg of root mass) was determined for each root sample.

2.6. Statistical analysis

Plant data were analysed using IBM SPSS statistics software (version 23). All data were first tested for normality and homogeneity of variances (Kolmogorov-Smirnov test & Levene's test). The time factor of the applied ANOVAs is not reported in all cases since its significance is obvious already from the graphs with the indicated SE values. The effect of fungal inoculation on plant dry weights, tissue P concentration and plant organ P content were therefore analysed per time point. If significant differences were found, a Tukey post-hoc test was applied. To correct for multiple comparisons in the one-sample *t*-tests applied, the outcomes were considered significantly different only at P values ≤ 0.0167 (*i.e.*, 0.05/3; Bonferroni correction for the three AMF inoculation treatments). All molecular analyses were conducted on log-transformed mtLSU DNA copy number data. Differences were considered significant at $P \leq 0.05$. The two P addition treatments were analysed separately because they were run sequentially in two consecutice experiments.

3. Results

3.1. Soil pH

A modest decrease of bulk pH in the sand substrate was recorded in the course of the orthophosphate experiment, irrespective of the AMF inoculation treatment: from 7.8 to 7.9 at wk0 to 7.5–7.6 at wk15. In the hydroxyapatite experiment, no obvious change in the pH of the substrate was observed in the non-mycorrhizal treatment, with pH values all lingering around 7.8 (± 0.05) at time points from wk3 onwards. In both mycorrhiza treatments the pH of the pot growth substrates gradually decreased with approximately 0.2 pH units ultimately.

3.2. Plant growth

When phosphorus was applied as orthophosphate there was no significant effect of myorrhyza on *P. lanceolata* biomass at final harvest (whole plant dry mass at wk15, Fig. 1a), nor on the course of vegetative growth as indicated by plant biomass at earlier time points. However, in the hydroxyapatite treatment, plant growth was significantly higher in the presence of *R. irregularis* than with *G. custos* or without AMF, at all time points, except wk3 (Fig. 1b). Factorial ANOVA (AMF inoculation treatment x time) on (non-)mycorrhizal plant dry matter accumulation across the 3–15 wk growth period showed, apart from a highly significant effect of time ($P < 0.001$; $F_{(4,\ 115)} = 451.2$), a highly

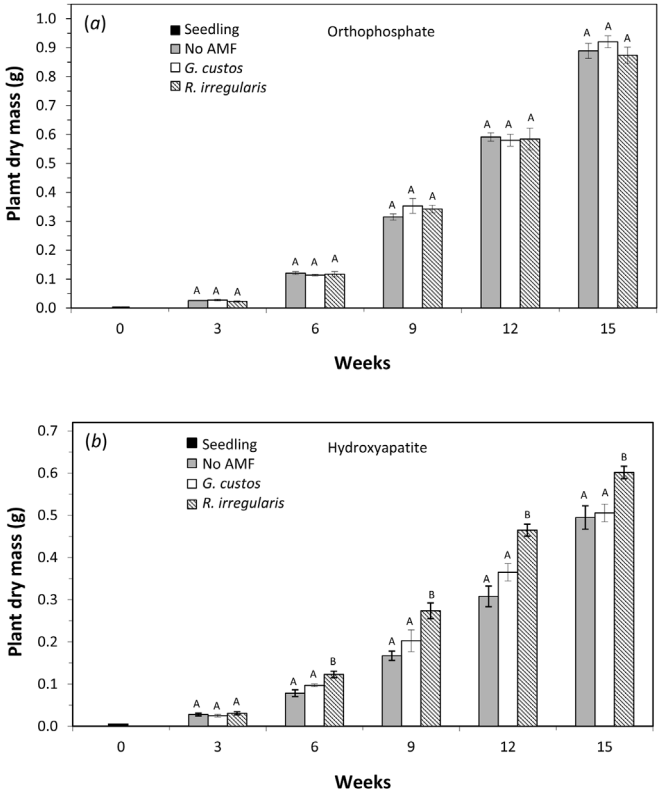

Fig. 1. Effects of arbuscular mycorrhizal fungal (AMF) species *Glomus custos* (white bars), *Rhizophagus irregularis* (hatched bars), and the absence of AMF (grey bars) on plant dry mass of *Plantago lanceolata* fertilized with a ½P-strength Hoagland nutrient solution (*a*), or growing in a sand substrate amended with hydroxyapatite (*b*). Values are shown as means ± SE of 7 (*a*) or 8 (*b*) replicates. Letters indicate significant differences among treatment means according to Tukey's HSD tests ($P \leq 0.05$) for each time point separately. The dark bars at t = 0 represent seedling dry mass before transplantation.

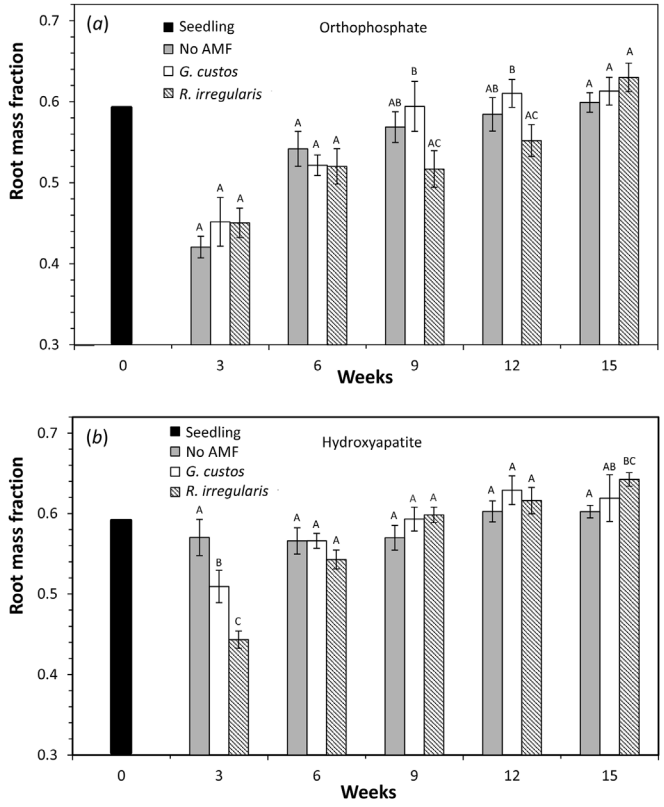

Fig. 2. Effects of arbuscular mycorrhizal fungal (AMF) species *Glomus custos* (white bars), *Rhizophagus irregularis* (hatched bars), and the absence of AMF (grey bars) on biomass allocation to roots of *Plantago lanceolata* fertilized with a ½P-strength Hoagland nutrient solution (*a*), or growing in a sand substrate amended with hydroxyapatite (*b*). Values are shown as means ± SE of 7 (*a*) or 8 (*b*) replicates. Letters indicate significant differences among treatment means according to Tukey's HSD tests ($P \leq 0.05$) for each time point separately. The dark bars represent biomass root allocation of seedlings before transplantation.

significant effect of AMF inoculation ($P < 0.001$; $F_{(2,\ 117)} = 32.6$). A Tukey post-hoc test indicated a significantly higher dry mass yield in the plants inoculated with *R. irregularis* compared to the 'no AMF' and '*G. custos*' treatments (both with $P < 0.001$), with no significant difference in dry mass accumulation between the latter two treatments ($P = 0.61$).

In addition to whole plant biomass we also analysed the fractional allocation to root tissues, as this is an important aspect of plant growth. At wk3 the root mass fraction was significantly lower than in the seedlings in all three AMF treatments ($P < 0.001$; one-sample *t*-tests, root/whole plant test value = 0.59), but it gradually increased during the course of the experiment (Fig. 2a). At the final harvest, the allocation mass to root did not differ between AMF treatments. However, when colonized by *R. irregularis* the host plant transiently changed the dry mass allocation in comparison with *G. custos,* allocating relatively more dry mass to its shoots (Fig. 2a, wk9 and wk12).

In the hydroxyapatite substrates, plant allocation to root at wk 3 was significantly different between all three AMF inoculation treatments, with the lowest root mass fraction measured in the *R. irregularis* treatment (Fig. 2b). The root fractions of the plant dry mass recorded in the presence of the fungi were also significantly different from the mass fraction in seedlings (i.e., $P = 0.005$ and $P < 0.001$ for *G. custos* and *R. irregularis*, respectively; *t*-tests with a root mass fraction test value of 0.59). However, like in the orthophosphate treatment, root mass fractions gradually increased to a value of around 0.62 towards the end of the experiment. This increase in the root mass fraction was highly significant in both inoculation treatments (one-way ANOVAs, each with $P < 0.001$). Across the entire growth period the fraction of dry matter allocated to roots in the non-mycorrhizal treatment did not change significantly (3–15wk period: one-way ANOVA, $P = 0.259$) and remained in the range of 0.57–0.60 (Fig. 2b).

3.3. Phosphate concentrations in root and shoot

In all treatments the phosphate concentrations in shoot seedlings were highest and a remarkable decrease in P concentration occurred in all three AMF inoculation treatments (Fig. 3). Root tissue P concentration of the seedlings was approximately half of that in the shoots, so in the roots the drop in P concentration was less pronounced (Fig. 4). In the orthophosphate substrates, the tissue P concentration of both shoot and root steadily decreased without consistent differences between the AMF treatments (Figs. 3a and 4a). No effects of AMF treatment on the (drop in) tissue P concentration were found apart from a relatively small but significant higher shoot P concentration at wk3 in the presence of *R. irregularis* (Fig. 3a).

For plants grown in hydroxyapatite substrate, shoot tissue P concentrations were significantly different among the AMF inoculation treatments, with the highest concentrations measured in the plants inoculated with *R. irregularis* (Fig. 3b). A sharp decrease in the P concentrations occurred in all AMF treatments over the first 3 weeks following seedling transplant (Fig. 3b), very similar to the rapidly dropping shoot P concentrations in the orthophosphate addition treatments (see Fig. 3a). This decrease of shoot P concentration was most pronounced with *G. custos* as symbiont, and least in the *R. irregularis*'s shoot P content (Fig. 3b). The sharp initial decline was followed by a further steady decrease in shoot P concentrations (one-way ANOVAs,

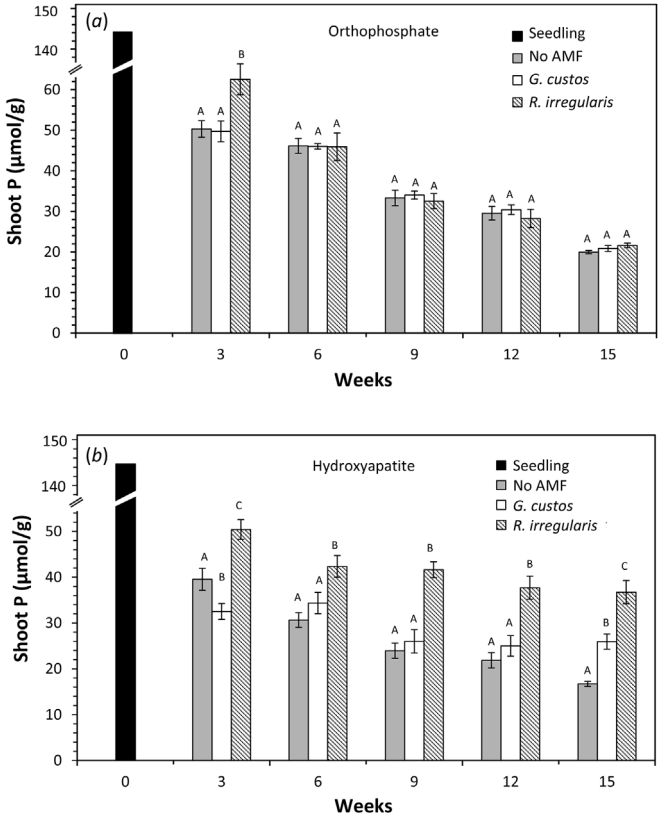

Fig. 3. Effects of arbuscular mycorrhizal fungal (AMF) species *Glomus custos* (white bars), *Rhizophagus irregularis* (hatched bars), and the absence of AMF (grey bars) on shoot P concentrations of the host plant *Plantago lanceolata* fertilized with a ½P-strength Hoagland nutrient solution (*a*), or growing in a sand substrate amended with hydroxyapatite (*b*). Values are shown as means ± SE of 7 (*a*) or 8 (*b*) replicates. Letters indicate significant differences among treatment means according to Tukey's HSD tests ($P \leq 0.05$) for each time point separately. The black bars represent the values measured in seedling shoots before transplantation.

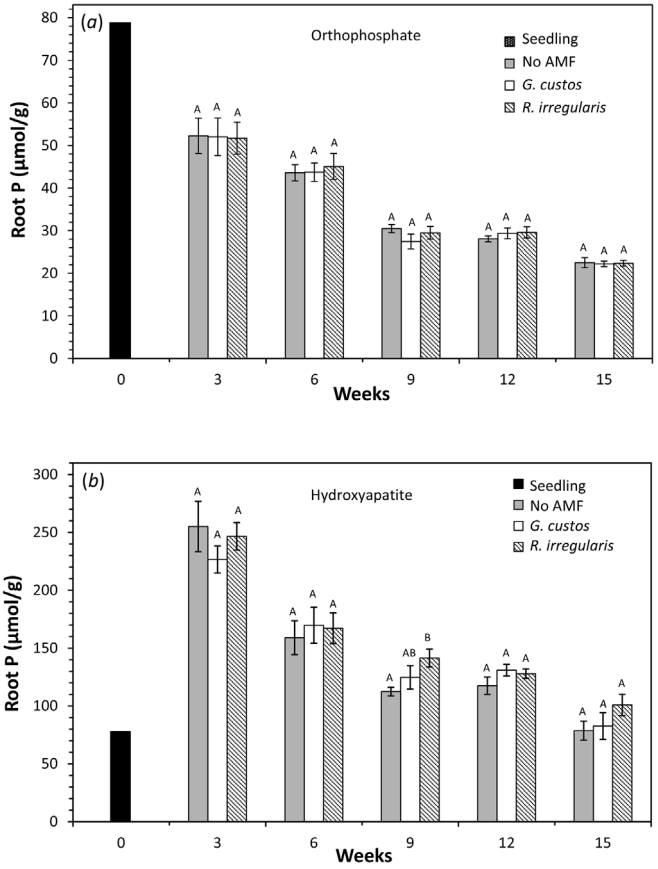

Fig. 4. Effects of arbuscular mycorrhizal fungal (AMF) species *Glomus custos* (white bars), *Rhizophagus irregularis* (hatched bars), and the absence of AMF (grey bars) on root P concentrations of the host plant *Plantago lanceolata* fertilized with a ½P-strength Hoagland nutrient solution (*a*), or growing in a sand substrate amended with hydroxyapatite (*b*). Values are shown as means ± SE of 7 (*a*) or 8 (*b*) replicates. Letters indicate significant differences among treatment means according to Tukey's HSD tests ($P \leq 0.05$) for each time point separately. The black bars represent the values measured in seedling roots before transplantation. Please note the difference of vertical scale between (*a*) and (*b*); the P concentrations in seedling roots are similar between (*a*) and (*b*). The data for roots grown in hydroxyapatite amended substrate may be influenced by hydroxyapatite crystals attached to the roots.

$P < 0.001$ and $P = 0.009$, respectively), with the steepest decline observed in the absence of AMF (Fig. 3b). A factorial ANOVA (AMF inoculation treatment x time) across the 3–15 wk period on (non-) mycorrhizal shoot P concentrations revealed highly significant effects of both time and treatment ($P < 0.001$ ($F_{(4,\ 115)} = 16.29$) and $P < 0.001$ ($F_{(2,\ 117)} = 50.74$), respectively). The Tukey HSD test indicated a significantly higher shoot P concentration in the plants inoculated with *R. irregularis* compared to the 'no AMF' and '*G. custos*' treatments (both with $P < 0.001$), with no significant difference in shoot P concentration between the latter two treatments ($P = 0.20$).

Plant total-P content (µmol P per plant) steadily increased from wk0 onwards, starting from ~0.27 µmol P per seedling, to approximately 19 µmol P per plant at wk15 (Supplementary data, Fig. S1a). No significant effects of the AMF inoculation treatments on plant total P acquisition were observed. A higher fraction of total-P was allocated to the shoots than to the roots in the seedlings (wk0, Fig. S1b), and in all the AMF treatments at wk3 (ratios total-P root/whole plant around 0.43, see Fig. S1b). However, steadily more P was allocated to the roots from wk6 onwards, eventually increasing the fraction of total-P in the roots to ~0.63 at wk15 (Fig. S1b). Between the AMF inoculation treatments, no significant differences in the allocation of total-P over root and shoot were found, apart from a transiently lower fraction of P in the roots at wk9 with *R. irregularis* as compared to the 'no AMF' treatment (Fig. S1b).

The P concentrations we measured in the roots of plants grown in hydroxyapatite-amended substrate were very high (Fig. 4b). These data are likely influenced by root-attached hydroxyapatite crystals which especially may have affected the measurements in very small plants (wk3).

Comparing the total shoot P content between the orthophosphate-P and apatite-P addition treatments, there were clear differences (shoot P content in Supplementary data, Fig. S2a and S2b, respectively). In orthophosphate-amended silica sand shoot P contents steadily increased from wk0 onwards (with ~0.16 µmol P per seedling shoot), but no significant effects of AMF inoculation treatment on shoot P content were observed. With hydroxyapatite-P, the shoot P contents increased over the entire period of incubation in all AMF treatments, but shoot P content in the *R. irregularis* treatment was more than double the shoot P contents recorded in the non-mycorrhizal treatment, and almost 70% more than in the presence of *G. custos* (Fig. S2b).

3.4. AMF root colonization

AMF abundance in roots as quantified by qPCR showed a steady and significant increase for both AMF species in orthophosphate-fertilized plants (Fig. 5a): one-way ANOVA *G. custos* mtLSU copy number ($P = 0.015$, $F_{(4,\ 31)} = 3.764$); one-way ANOVA *R. irregularis* mtLSU copy number ($P = 0.017$, $F_{(4,\ 32)} = 3.639$). From wk3 to wk12 mtLSU

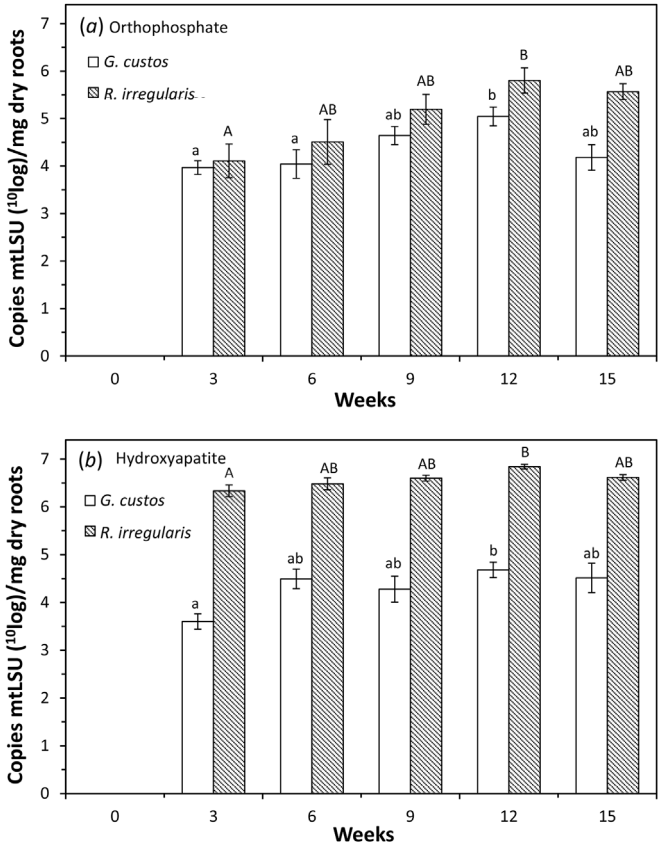

Fig. 5. Root colonization (expressed as AMF mtLSU copy number abundance) by *Glomus custos* (white bars), and *Rhizophagus irregularis* (hatched bars) of *Plantago lanceolata* fertilized with (*a*) ½P-strength Hoagland solution or (*b*) hydroxyapatite-P. Copy numbers are shown as means ± SE of 7 (panel *a*) or 8 (panel *b*) replicates. Letters above the bars indicate the significance of the time effect on colonization according to Tukey's HSD tests ($P \leq 0.05$) for *G. custos* (lowercase letters), and *R. irregularis* (uppercase letters), separately.

copy numbers of *G. custos* and *R. irregularis* increased approximately 12- and 50-fold, respectively. Thereafter, at wk15 mtLSU copy numbers of the fungi showed a not significant decrease (Fig. 5a). qPCR on non-mycorrhizal roots ('no AMF' control treatment) showed the absence of (cross)infections by *G. custos* and/or *R. irregularis* (not shown), and direct microscopic inspection did not provide any indication of a colonization of the roots by infecting extraneous AMF. Likewise in the mycorrhizal treatments qPCR demonstrated the absence of an unwanted crossinfection event caused by either one of the two AMF.

In the plants grown in apatite-amended silica sand, the mtLSU copy numbers also increased significantly from wk3 to wk12 with a non-significant decrease again at wk15 (Fig. 5b), according to one-way ANOVA for *G. custos* mtLSU copy number ($P = 0.022$, $F_{(4, 37)} = 3.313$), and one-way ANOVA for *R. irregularis* mtLSU copy number ($P = 0.004$, $F_{(4, 39)} = 4.645$). In this period the *G. custos* mtLSU copy number increased 12-fold, whereas the increase in copy number in the *R. irregularis* treatment now was significantly less, only ~ twofold. However, already at wk3 the *R. irregularis* mtLSU copy number amounted to 2.16×10^6 copies/mg dry root, *i.e.*, almost 170-fold the copy number measured in the orthophosphate-fertilized plants at wk3 (Fig. 5a). Similar to the plants in the orthophosphate addition treatment qPCR and direct microscopic root inspection did not indicate the occurrence of AMF infection(s) of host roots in the non-mycorrhizal treatment, or the occurrence of cross-infections over treatments.

4. Discussion

Using a detailed time-course approach, we found that the arbuscular mycorrhizal fungus *Rhizophagus irregularis* provided a far greater delivery of phosphorus to the plant than *Glomus custos* when *Plantago lanceolata* was grown in hydroxyapatite-amended silica sand. We observed more than 90% difference in shoot P content between the two AMF species. This is much higher than the *c*. 24% difference in shoot P content found previously [30] when a readily soluble orthophosphate was used as a P source. Given the pH (~7.2) and calcium richness of the calcareous dune sand used in previous studies [13,30], the presence of apatite-like mineral P species in their sand seems likely, and even more so because the added orthophosphate is prone to precipitate with calciumcarbonate in the dune sand [45]. We therefore assume that the higher symbiont quality of *R. irregularis* observed in previous work [13,30,46,47], like in the present study, was brought about by the superior ability of this AMF species to solubilize apatite-like minerals in comparison to *G. custos*.

Our studies were conducted in a climate room allowing precise temperature and humidity control, but providing only modest lighting of 175–185 μmol m^{-2} s^{-1} PAR. This amount of light is appreciably less (2–5x) than normally available in a greenhouse given average light conditions during the growing season (spring/summer) in the Netherlands. Given the equal P status and very similar growth characteristics of the AMF-colonized and the non-mycorrhizal plants in our experiment, we conclude that the availability of orthophosphate, despite its reduction in the applied Hoagland solution, was not limiting because growth of the host plant, most likely, was mostly limited by light. The light regime may influence the response of plants to mycorrhiza [48]. Nonetheless, the qPCR recordings indicated a substantial intraradical growth of both AMF species. AMF root colonization under conditions where plant delivery of P to the plant is not necessary, has been reported previously [49].

In the early growth stage dry matter allocations to roots differed significantly between the three AMF inoculation treatments. In the absence of AMF the hydroxyapatite apparently necessitated a higher initial investment by the plant in its root system [50]. Indeed, in the inoculation treatments with *R. irregularis* and *G. custos* the root mass fractions (range: 0.44–0.50) were quite similar to those observed at wk3 in the orthophosphate-fertilized plants, suggesting a relatively less stringent limitation of growth by P in this period. In most plants species, seeds contain sufficient P to support growth of the seedling for several weeks after germination [51], with concomitant strong dilution of the shoot and root tissue P concentrations as a result. The initial, transient higher investments in shoot mass can be advantageous for seedlings and young plants experiencing low irradiation levels when there is high competition for available light for example because of shading neighbor plants. Likewise in the orthophosphate-fertilized plants, the temporary higher allocation of dry mass to the shoots in the '*R. irregularis*' treatment in comparison with *G. custos*-inoculated plants, may also be a physiological response to competitive environments characterized by low incident light levels.

An interesting observation from our experiment is the ability of *P. lanceolata* to solubilize P from a resilient crystalline mineral matrix even in the absence of mycorrhizae. To our knowledge there are only few similar observations on direct root dissolution/utilization of apatite-P in the literature [52]. In natural environments the release of phosphorus by mineral weathering processes is thought to be the result of the concerted activities (*i.e.*, rhizosphere acidification, the excretion of chelators/ligands/enzymes) of plant roots and the many different soil microorganisms also present [29,53,54]. We are aware of only two studies on the capability of AMF to mobilize phosphorus from apatite under defined monoxenic culture conditions. Using a root-free hyphal compartment, *Glomus manihotis* was shown to be able to solubilize P from an apatite matrix, thus significantly promoting the uptake of phosphate rock-derived P by maize [25]. A root organ culture of *R.*

irregularis strain DAOM 197198 was also able to release P from an apatite matrix [29]. In our P-addition experiments host roots and ERM were not spatially separated, thus allowing synergistic activities to occur between hyphae and roots in P mobilization and uptake, but recent experiments with hydroxyapatite only available in a root-free compartment showed that both *R. irregularis* and *G. custos* ERM were able to sustain growth of *P. lanceolata*.

The P-solubilizing action of AMF does not come with appreciable changes of soil pH. Only a very modest decrease in pH of the apatite-amended (non-buffered) growth substrate occurred in the AMF inoculation treatments: a lowering pH 0.2 units at still alkaline values. In this respect, the AMFs in our study bear some resemblance with the biogeochemically active 'non-acidifying' ascomycete grassland isolates in the weathering process of fluorapatite crystals [55]. Their ANG74 strain was capable of sequestering apatite particles within mycelial spheres (in liquid culturing), suggesting an apatite dissolution mechanism by means of intimate ligand-surface interactions without the excretion of large amounts of organic acids. In contrast, the examined zygomycete soil isolates showed a fluorapatite weathering with concomitant (strong) medium acidification and substantial organic acids excretions (final pH ranges: 4.9–3.3). Monitoring of the accumulation of carboxylate exudates in our silica growth substrate (low molecular weight acids, *e.g.* citric, oxalic, (keto)gluconic acids) is required for a better understanding of the apatite-mobilizing mechanism(s) possibly employed by our AMF and/or *P. lanceolata* [29,50,56].

An unexpected result from the hydroxyapatite addition experiment is the low shoot and root dry mass yield of the *R. irregularis*-colonized plants relative to the yields measured in the orthophosphate-fertilized host. As the total P accumulation recorded in the shoots of the apatite-P/*R. irregularis* treatment was comparable to shoot P content observed in the orthophosphate-grown plants, a factor other than the hydroxyapatite alone must have responsible for constraining the gain in (shoot) dry mass of the host plant in this treatment. A possible explanation could be that despite the sufficient availability of phosphorus, light formed a limiting (co-)factor for growth. This explanation assumes that the carbon costs to maintain the highly beneficial association with *R. irregularis* forced the host to reduce the allocation of photosynthates to the shoots and root system under the low light conditions present. Support for this idea originates from the consistently high mtLSU copy number of *R. irregularis* in the hydroxyapatite treatment, strongly suggesting immediate and high investments for the required build-up of the IRM and ERM networks. In the orthophosphate-grown plants, mtLSU copy number of this AMF was almost 170-fold lower during the initial weeks of the experiment, which is indicative for the potentially large differences in incurred carbon costs for the host depending on the source of P. Co-limitations of plant growth by phosphorus and nitrogen have been reported frequently in the literature [57] but far less so for co-limitations involving light and mineral nutrients. The only reports we are aware of are studies on early growth of maritime pine seedlings showing the co-limitation by light and P, and a combined field/greenhouse study describing the co-limitation by light and soil nutrients of aspen seedlings, [58,59].

The carbon costs involved for *P. lanceolata* in the colonization of its roots by AMF are not easily quantifiable. Fungal abundance expressed in mtLSU copy number per unit root (dry) mass is a mixed proxy for both standing IRM biomass and hyphal activity/vitality [60,61]. The conversion therefore, of these numbers into fungal carbon investments, and thus the related incurred costs for the host plant, is by no means straightforward, and to our knowledge no attempt of such a conversion has been reported to date. Nevertheless, an indirect and first approximation of these costs can be conjectured in the case of *R. irregularis* as the symbiotic partner when comparing the orthophosphate- and the apatite-P-grown plants. The highly beneficial cooperation of *P. lanceolata* and this AMF when utilizing hydroxyapatite as P source may have called for carbon investments by the host plant encompassing up to some 20% of its photosynthetic capacity, the percentage often stated in the literature to illustrate significance and (potential) magnitude of carbon allocation to the symbiont(s) in mycorrhizal plants [8,62]. So at wk12 with Hoagland-P as source of phosphorus, the ~10-fold lower abundance in mtLSU copy number of *R. irregularis* compared to the copy number in the apatite treatment (highest copy numbers at wk12 in both P-addition treatments, would correspond to some 2% of the carbon fixed in host photosynthesis, maximally: *i.e.*, a relatively modest investment despite the roughly 50-fold increase in copy number in this treatment from wk3 onwards. A similar comparative assessment of incurred costs for *P. lanceolata* in association with *G. custos* is not as straightforward because, quite surprisingly, mtLSU copy numbers recorded at most of the time points in the orthophosphate treatment were higher than those in the apatite-P addition experiment, suggesting that IRM proliferation in this AMF/host combination is not strongly dependent on the nature of the source of P utilized for growth (but see Ref. [49] for a review on various reported AMF responses and related host 'carbon economy'). Possibly, the resilient hydroxyapatite called for a relatively much stronger development of its ERM than IRM in *G. custos* in comparison to *R. irregularis,* due to differences in apatite-weathering capabilities of the two AMF (*e.g.* related to the amounts of exuded organic acids/ligands [55,63]). The allocation ratio of carbon over ERM and IRM is of much importance in this respect since a major portion of the photosynthate expenditure associated with root colonization is used in the formation of external hyphal networks [8].

5. Conclusions

Mixing crystalline hydroxyapatite into quartz sand was a simple and effective way to create P-limiting conditions under well-defined growth substrate and mineral nutrient conditions. This possibility warrants conduction of further growth trials, using other poorly soluble P-minerals, such as $Ca_3(PO_4)_2.nH_2O$, $CaHPO_4.2H_2O$ or $Ca_8H_2(PO_4)_{6.5}H_2O$. These relatively stable (amorphous) P-species reflect different stages in orthophosphate/calcium precipitation under neutral/alkaline soil conditions [4,35], thus possibly enabling a further characterization of the P dissolution abilities of AMF.

Besides dry mass yield and host total P acquisition, other plant growth characteristics such as shoot-to-root dry mass allocation and shoot tissue P concentration also reflected the functional differences between the AMF species *R. irregularis* and *G. custos*, during the very early or the intermediate developmental stages of the symbiosis. The initial and intermediate stages of root colonization are particularly useful in the delimitation of mycorrhizal traits of different AMF species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ejsobi.2018.07.004.

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